SUPPLEMENTARY INFORMATION FOR

Increased Robustness of Single-Molecule Counting with Microfluidics, Digital Isothermal Amplification, and a Mobile Phone versus Real-Time Kinetic Measurements

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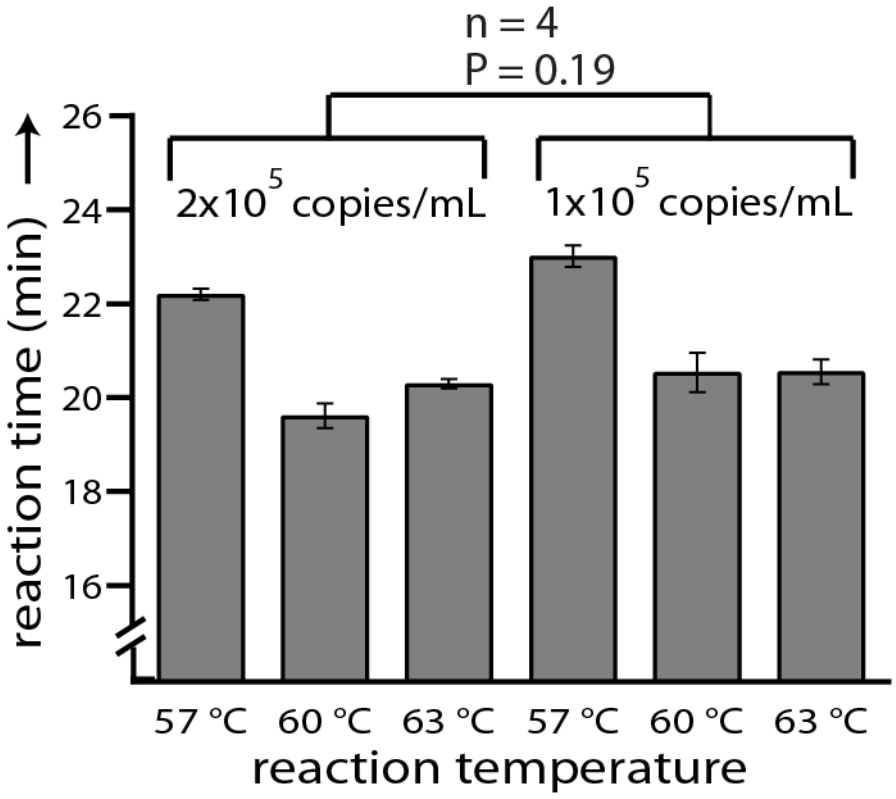
Supplementary Figure S1 A graph showing the results of one-step real-time RT-LAMP experiments for two concentrations at three temperatures across a 6-degree temperature range. The one-step real-time RT-LAMP reaction could not successfully distinguish the two concentrations in the presence of temperature fluctuations. P values denote statistical significance of all data for each concentration at a given imaging condition, irrespective of temperature (the null hypothesis being that the two concentrations were equivalent). Imaging was performed with a microscope. Error bars represent standard deviation.



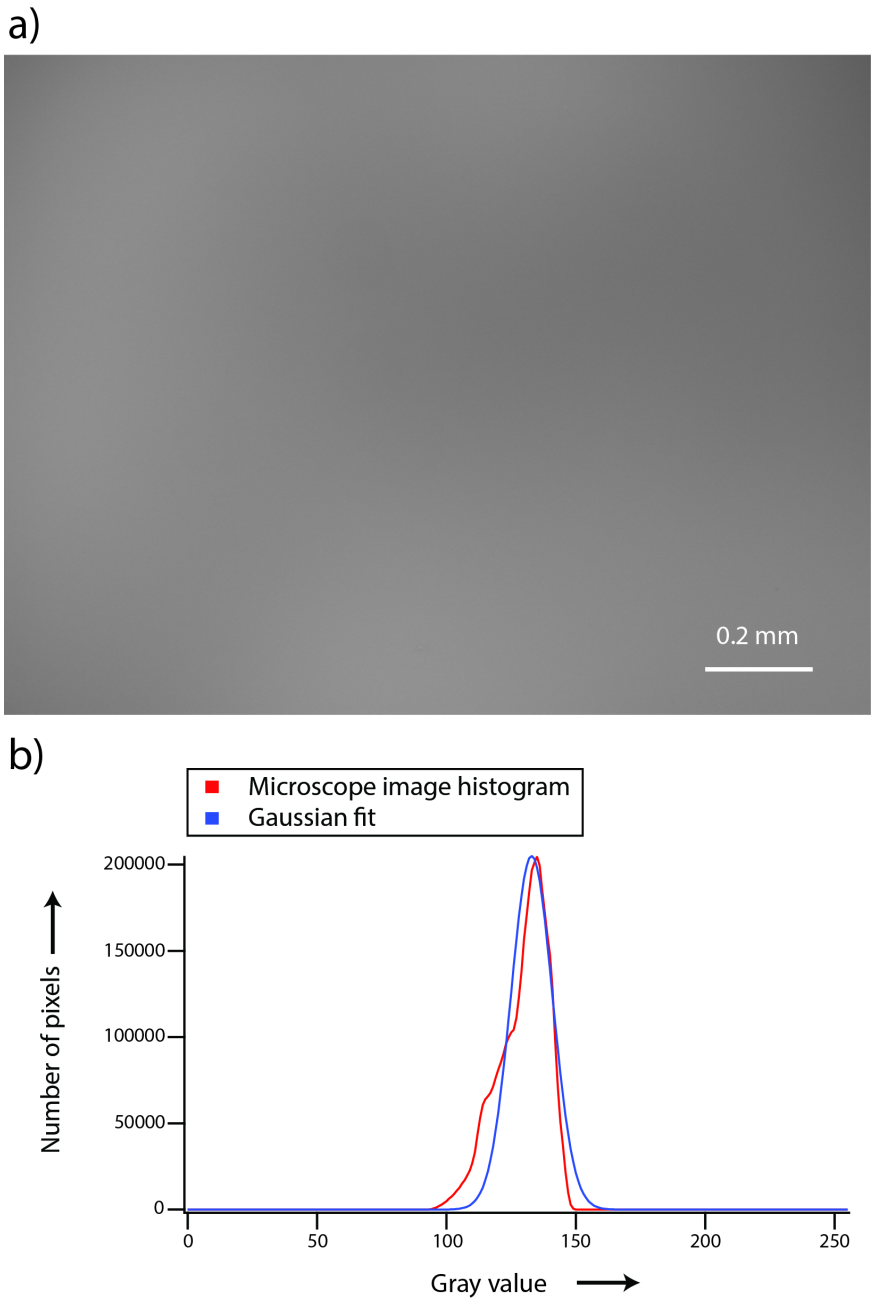
Supplementary Table S1 Sequences of primers used for RT-LAMP amplification of HIV viral RNA selected from a previous publication[1] with several single point mutations.

p24 Loop B	GAG AAC CAA GGG GAA GTG A
p24 Loop F	TTT AAC ATT TGC ATG GCT GCT TGA T
p24 BIP	TAT TGC ACC AGG CCA GAT GAT TTT GTA CTA GTA GTT CCT GCT ATG
p24 FIP	CAG CTT CCT CAT TGA TGG TCT CTT TTA ACA CCA TGC TAA ACA CAG T
p24 F3	ATT ATC AGA AGG AGC CAC C
p24 B3	CAT CCT ATT TGT TCC TGA AGG

Supplementary Table S2 Layout of primers on the multiplexed PCR device (Figure 3)

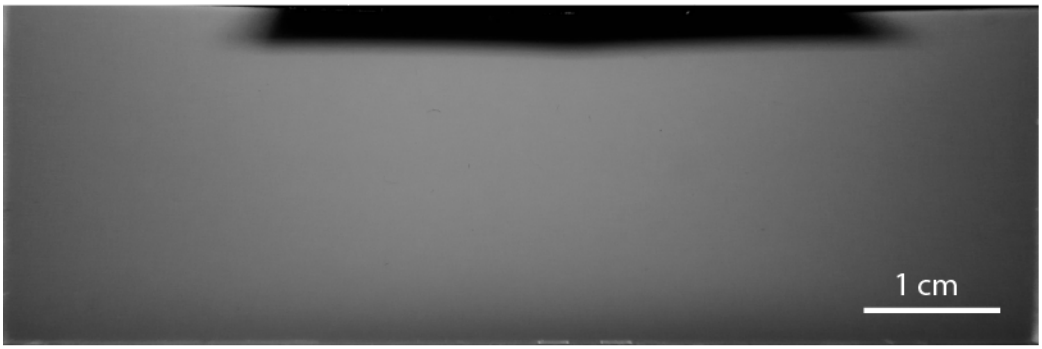
Position	Name of Primer Set	Primer Sequence
1	*Escherichia coli* nlp gene	Forward: ATA ATC CTC GTC ATT TGC AG Reverse: GAC TTC GGG TGA TTG ATA AG
2	*Pseudomonas aeruginosa* vic gene	Forward: TTC CCT CGC AGA GAA AAC ATC Reverse: CCT GGT TGA TCA GGT CGA TCT
3	*Candida albicans* calb gene	Forward: TTT ATC AAC TTG TCA CAC CAG A Reverse: ATC CCG CCT TAC CAC TAC CG
4	*Pseudomonas* 16S	Forward: GAC GGG TGA GTA ATG CCT A Reverse: CAC TGG TGT TCC TTC CTA TA
5	*Staphylococcus aureus* nuc gene	Forward: GCG ATT GAT GGT GAT ACG GTT Reverse: AGC CAA GCC TTG ACG AAC TAA AGC

Supplementary Figure S2 Uniformity analysis of images taken with a microscope. a) An image used to analyze the uniformity of illumination and emission that result from a microscope imaging setup. A fluorescent slide was imaged with a microscope under the same conditions used to image dRT-LAMP reactions. Exposure time was 50 ms for dRT-LAMP reactions and 3 ms for the image below. The original 12-bit image was collapsed into an 8-bit image so that it could be directly compared to the image of the cell phone. b) A histogram of the image in (a) with a Gaussian fit of the data. The full width at half maximum (FWHM) of the Gaussian curve is 18.9. Thus, the illumination field was relatively even with a tight distribution.

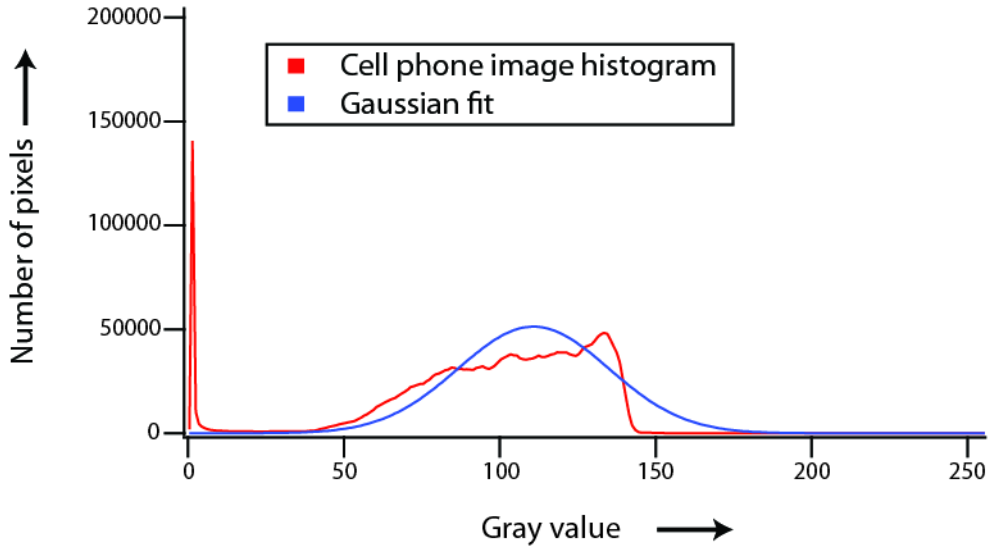


Supplementary Figure S3 Uniformity analysis of images taken with a cell phone camera. a) An image used to analyze the uniformity of illumination and emission that result from imaging with a cell phone and a shoe box. The image was obtained by taking an RGB color photograph of a fluorescent slide and removing the red and blue channels from the image, thus retaining only the green channel. The camera settings were ISO 800 with the exposure number set at -2.0. The image was corrected for lens distortion. b) A histogram plot of the image in (a) with a Gaussian fit of the data. The FWHM of the Gaussian curve is 57.0. The Gaussian models only the second peak, as the first peak is caused by the shade on the top of the image. As can be seen below, the illumination field is subject to larger variations than those experienced in the microscope imaging setup (Supplementary Figure S2).

a)



b)



Chemicals and materials

All solvents and salts purchased from commercial sources were used as received unless otherwise stated. The LoopAmp DNA amplification kit, the LoopAmp RNA amplification kit, and the Calcein fluorescence indicator kit were purchased from SA Scientific (San Antonio, TX, USA). SsoFast EvaGreen Supermix (2X) was purchased from Bio-Rad Laboratories (Hercules, CA). Bovine serum albumin (BSA) was purchased from Roche Diagnostics (Indianapolis, IN). Hybridase Thermostable RNase H was purchased from Epicentre Biotechnologies (Madison, WI). All primers were ordered from Integrated DNA Technologies (Coralville, IA). Mineral oil (DNase-, RNase-, and Protease-free), tetradecane, and DEPC-treated nuclease-free water were purchased from Fisher Scientific (Hanover Park, IL). Dichlorodimethylsilane was purchased from Sigma-Aldrich (St. Louis, MO). AcroMetrix HIV-1 Panel Copies/ml, EXPRESS One-Step SYBR GreenER Universal, iPrep purification instrument, and iPrep PureLink virus kit were purchased from Life Technologies (Grand Island, NY). PCR Mastercycler was purchased from Eppendorf (Hamburg, Germany). Eco real-time PCR system was purchased from Illumina, Inc. (San Diego, CA). Photomasks were designed in AutoCAD 2013 and ordered from CAD/Art Services, Inc. (Bandon, OR). Soda-lime glass plates coated with layers of chromium and photoresist were ordered from the Telic Company (Valencia, CA). Genomic DNA (*Staphylococcus aureus*, ATCC number 6538D-5) was purchased from American Type Culture Collection (Manassas, VA).

Fabrication of SlipChips

The procedure for fabricating the SlipChips for both the dRT-LAMP and multiplexed PCR experiments were based on previous work[2]. The dRT-LAMP devices were fabricated using a two-step exposing-etching protocol that was adapted to create wells of two different depths (5 μm for thermal expansion wells and 55 μm for all the other wells in the dRT-LAMP device; 40 μm for the thermal expansion wells and 75 μm for all other wells in the multiplexed PCR device). After etching, both devices were subjected to the same glass silanization process, where the glass plates were first thoroughly cleaned with piranha mix and dried with 200 proof ethanol and nitrogen gas, and then oxidized in a plasma cleaner for 10 minutes and immediately transferred into a vacuum desiccator for 1.5 hours for silanization with dimethyldichlorosilane.

After silanization, the devices were rinsed thoroughly with chloroform, acetone, and ethanol, and dried with nitrogen gas before use. When a glass SlipChip needed to be reused, it was cleaned with Piranha acid first, and then subjected to the same silanization and rinsing procedure described above.

SlipChip device design

The design of the SlipChip device used in this paper was the same as a previously published SlipChip design, with slight modification[3]. The device was modified to include four etched circles that direct the placement of the four red alignment markers. The device contained a total of 1,280 wells (each with a volume of 6 nL) on either half of the chip; however, when the two halves were manipulated to combine the reagents and initiate reactions, only 1,200 individual reactions were initiated.

Assembling and loading the SlipChips

The SlipChips for both the dRT-LAMP and multiplexed PCR experiments were assembled under degassed oil (mineral oil: tetradecane 1:4 v/v for dRT-LAMP and pure mineral oil for PCR). Both top and bottom plates were immersed into the oil phase and placed face to face. The two plates were aligned under a stereoscope (Leica, Germany) and fixed using binder clips. Through-holes were drilled into the top plate to serve as fluid inlets and oil outlets in dead-end filling. The reagent solutions were loaded through the inlets by pipetting.

dRT-LAMP amplification of HIV-1 RNA on SlipChip

We used a previously described HIV-1 viral RNA purification protocol from AcroMetrix HIV-1 Panel Copies/mL[3]. The first solution, which was used for amplifying HIV-1 RNA using the two-step dRT-LAMP method, contained the following: 10 μL RM, 1 μL BSA, 0.5 μL EXPRESS SYBR GreenER RT module (part of EXPRESS One-Step SYBR GreenER Universal), 0.5 μL BIP primer (10 μM), various amounts of template, and enough nuclease-free water to bring the volume to 20 μL. The second solution contained 10 μL RM, 1 μL BSA, 2 μL EM (from LoopAmp RNA amplification kit), 1 μL or 2 μL FD, 2 μL other primer mixture (20 μM FIP, 17.5 μM FIP, 10 μM LooP_B/Loop_F, and 2.5 μM F3), 1 μL Hybridase Thermostable RNase H, and enough nuclease-free water to bring the volume to 20 μL. The first solution was loaded onto

a SlipChip device and incubated at 50 °C for 10 min, and then the second solution was loaded onto the same device and mixed with the first solution. The entire filled device was incubated at various temperatures (57 °C, 60 °C, or 63 °C) for 60 minutes.

Real-time RT-LAMP amplification of HIV-1 RNA

The purified HIV-1 RNA described above was used for real-time in-tube RT-LAMP amplification. For two-step RT-LAMP amplification, a first solution containing the reagents described above was first incubated at 50 °C for 10 min and then mixed with a second solution, as described above. The 40 µL mixture was split into 4 aliquots and loaded onto an Eco real-time PCR machine. The amplification was performed at various temperatures (57 °C, 60 °C, or 63 °C) for 60 min. For one-step RT-LAMP amplification, the RT-LAMP mix contained the following: 20 µL RM, 2 µL BSA (20 mg/mL), 2 µL EM, 2 µL FD, 2 µL of primer mixture (20 µM BIP/FIP, 10 µM LooP_B/Loop_F, and 2.5 µM B3/F3), various amount of template solution, and enough nuclease-free water bring the volume to 40 µL. The 40 µL mixture was split into 4 aliquots and loaded onto the Eco real-time PCR machine. The amplification was performed at various temperatures (57 °C, 60 °C, or 63 °C) for 60 min. Data analysis was performed using Eco software. To determine the reaction time (time required for the fluorescent signal to cross the threshold), fluorescence intensity between 5 min and 15 min was used as the baseline and the threshold value was set to be 2.

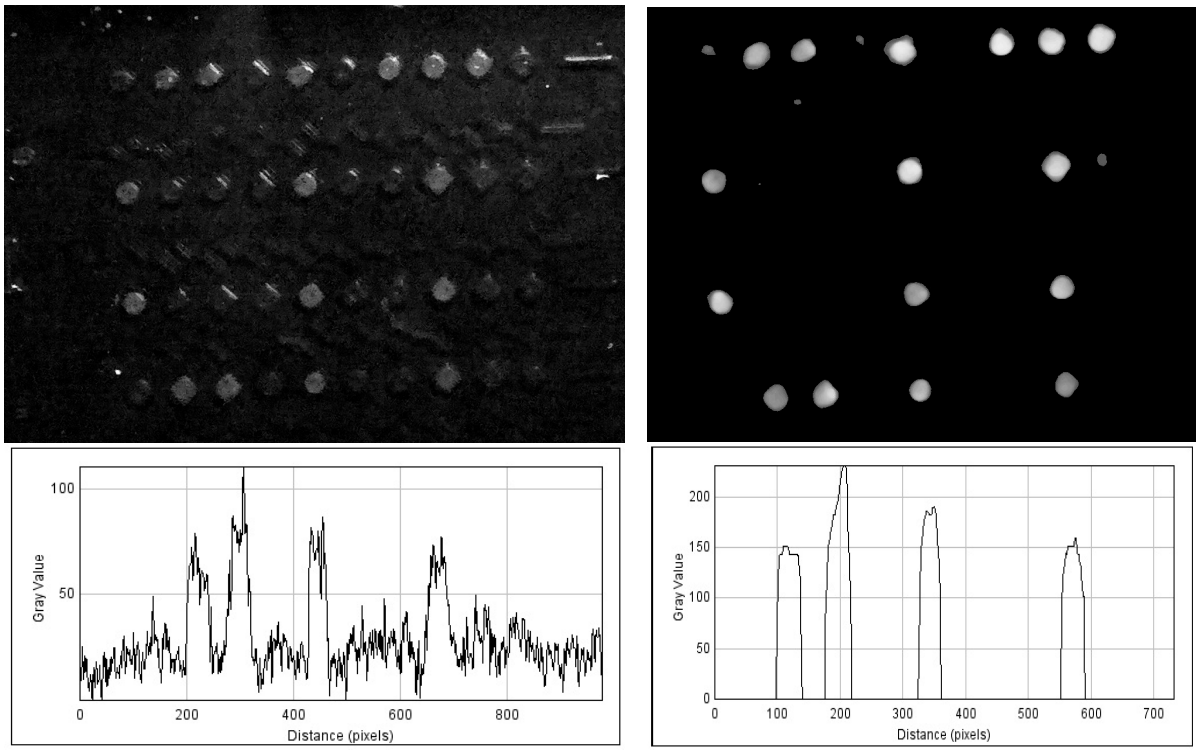
Multiplexed PCR amplification on SlipChip

The PCR mixture used for amplification of *Staphylococcus aureus* genomic DNA on a multiplexed SlipChip contained the following: 10 µL 2X SsoFast Evagreen SuperMix, 1 µL of 20 mg/mL BSA, 1 µL of 1 ng/µL gDNA, 0.5 µL SYBR Green (10x) and 7.5 µL RNase-free water. Primers were pre-loaded onto the chip using a previously described technique.[2] Briefly, each primer pair described in Table S1 was deposited into different circular expansion wells of the SlipChip at a concentration of 0.15 µM and a volume of 0.1 µL, using Teflon tubing (200 µm inner diameter) connected to a 50 µL Hamilton glass syringe. The solution was allowed to dry at room temperature overnight, and the preloaded SlipChip was used the following day. The PCR amplification was performed with an initial 95 °C step for 5 min, and then followed by 40 cycles of: (i) a DNA denaturation step for 1 min at 95 °C, (ii) a primer annealing step of 30 sec at

55 °C, and (iii) a DNA extension step of 45 sec at 72 °C. An additional 5 min at 72 °C was performed to allow thorough dsDNA extension, and the SlipChip was kept in the thermocycler at 4 °C before imaging.

Cell phone imaging and image processing for multiplexed PCR devices

Cell phone imaging was performed with a painted shoebox, as described below. All images were taken using the standard cell phone camera application. The white balance was set to automatic, the ISO was set at 1,600, the exposure value was set at +4, the focus mode was set to “close-up,” and the resolution was adjusted to 8 MP. Images were processed using a free Fiji image processing package available on the Internet according to the standard procedure: first, the color channels of the original image were split; second, the red channel was subtracted from the green channel to eliminate scattering artifacts (this procedure makes the area surrounding positive wells essentially negative, or equal to 0); third, a median filter with a radius of 9 pixels was applied to the subtracted image; fourth, the “enhance contrast” option was applied with “equalize histogram” checked. Finally, the image was cropped and colored green to indicate the green channel. The original cropped green channel image and the processed image, along with cross-sections (also made on Fiji) of the four bottom wells in both, are shown below (Supplementary Figure S4):



Supplementary Figure S4 Top: Images of the device with PCR reaction outcomes taken by a cell phone before (left) and after (right) image processing. Bottom: Line scans showing gray values as a function of distance in pixels are shown for each image. Image processing procedure is described in the text.

Microscope image acquisition and analysis

Fluorescence images of each device were acquired using a Leica DMI 6000 B epi-fluorescence microscope with a 5X / 0.15 NA objective and L5 filter at room temperature. All the images were analyzed using MetaMorph software (Molecular Devices, Sunnyvale, CA). Images taken in each experiment were stitched together and a dark noise background value of 110 units was subtracted before the image was thresholded. The number of positive wells was automatically counted using the integrated morphology analysis tool based on intensity and pixel area. The concentrations of HIV-1 RNA were calculated based on Poisson distribution, as described in a previous publication[4].

Typical arbitrary fluorescence values for the negative wells were 80 ± 10. Arbitrary fluorescence values for the positive wells were largely centered around 350 ± 100.

Cell phone camera setup and settings

A Nokia 808 Pureview cell phone was used to image and count microwells containing the amplification product. This cell phone features a CMOS sensor with a Xenon flash. The Nokia 808 uses a 1/1.4-inch 41-megapixel sensor with a pixel size of 1.4 μm. The camera has a Carl Zeiss F2.4 8.02 mm lens and contains a set of "PureView" modes which are able to combine multiple pixels by using pixel oversampling, thus increasing an individual pixel's area (which, consequently, lowers the image's resolution). This feature increases the sensitivity of each individual pixel in the final image.

Since the shortest focal length of the camera's lens is 15 cm, we used a non-branded, commercially available 0.67 x magnetically mounted wide lens designed for iPhone. Using this objective, we were able to obtain images at a distance of 6.5 cm from the device, which effectively increased the total number of photons collected from the device, thus improving camera sensitivity.

A set of filters was used for both excitation and emission purposes. To improve filtering, one additive dichroic filter (model number FD1B, Thorlabs, Newton, NJ) was cut in half and the two halves were stacked and attached to the camera. For fluorescence detection, two 5CGA-530 long-pass filters (Newport, Franklin, MA) were inserted into magnetically mounted lens.

The imaging process with a cell phone was performed with the device tilted at 10 degrees relative to the cell phone plane to prevent direct reflection of the flash to the lens. This was achieved by placing the device on a tilted glass slide (3" x 2") that had been painted black on the bottom side to reduce background noise. Tetradecane was placed in between the device and the slide to reduce glare.

All images were taken using the stock camera application. The white balance was set to automatic, the ISO was set at 800, the exposure value was set at +2, the focus mode was set to "close-up," and the resolution was adjusted to 8 MP.

Real-time dRT-LAMP imaging and analysis

Time traces of dRT-LAMP reactions were obtained by imaging a progressing dRT-LAMP reaction every minute using a Leica MZFLIII stereomicroscope. This was performed by placing

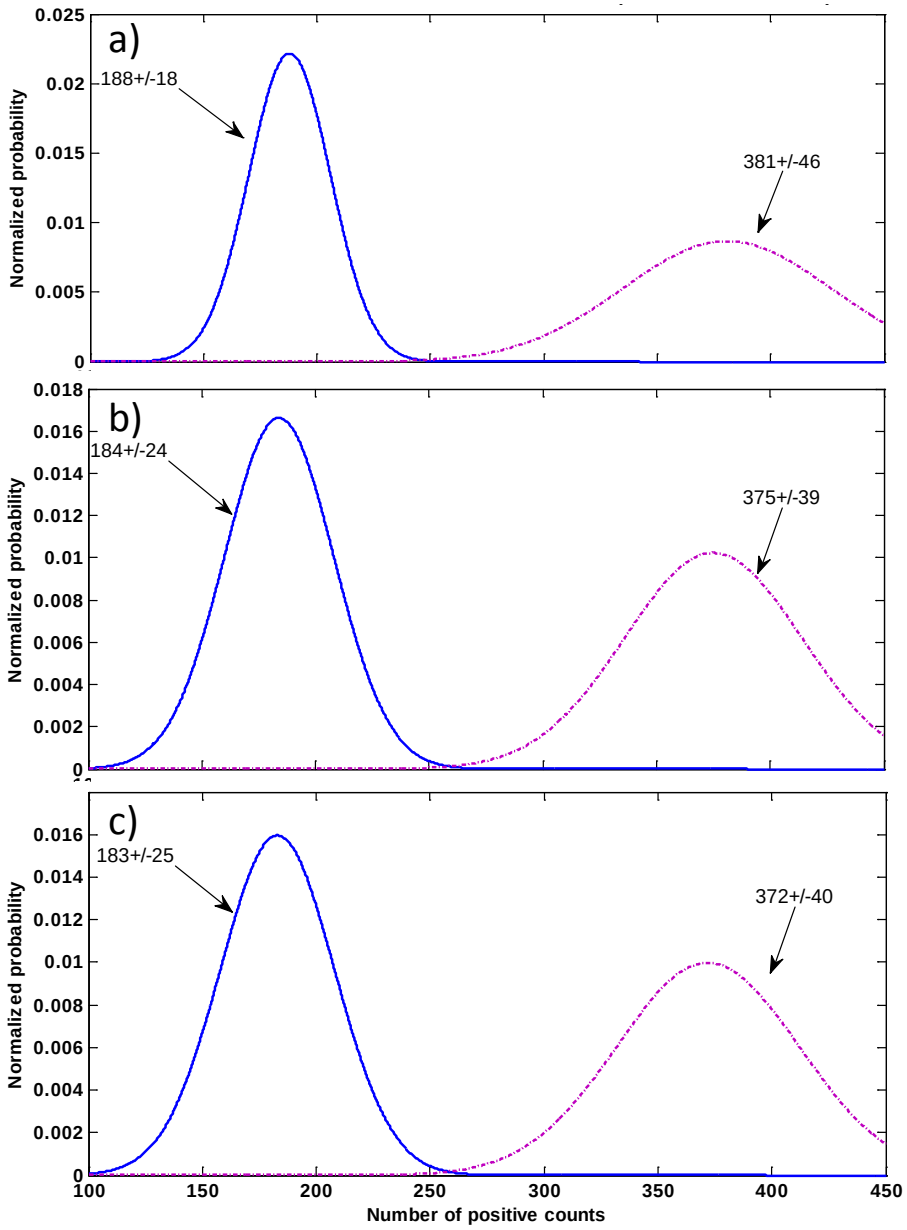
an Eppendorf mastercycler with an in-situ adapter plate within the imaging field of the stereoscope. The stereoscope was set to a magnification of 1X and placed in such a way that it could move relative to the position of the thermocycler. At each required time-point, a fluorescent image of one-half of the device was taken, and then the stereoscope was moved to image the second half of the device. The camera attached to the stereoscope was a Diagnostic Instruments color mosaic model 11.2 camera and images were acquired using Spot imaging software. The imaging was done with a gain of 4 and an exposure time of 2 seconds. The acquired images were then analyzed using the ImageJ software package. The images were manually edited to remove wells that appeared in both images, after which, the green color plane was extracted and a threshold value of 30 was applied. The binary image was then filtered to keep all spots within an area between 65 and 350 leaving the number of positive wells. After performing this analysis with images comprising both halves of the device, the numbers for each half were added together, yielding the total number of positive wells at that specific time point.

Statistical analysis of data sets obtained at different temperatures

The t-test is used to evaluate whether the means of two different data sets are statistically different. The p value obtained in this process is the probability of obtaining a given result assuming that the null hypothesis is true. A 95% confidence level, which corresponds to $p = 0.05$, or a 5% significance level, is commonly acceptable. It is typically assumed that the concentrations of two samples are different when $p < 0.05$. Here, we used a p value to evaluate the performance of two-step dRT-LAMP in various imaging conditions—with a microscope, with a cell phone and a shoe box, and with a cell phone in dim lighting. When we pooled all data for one concentration from different temperatures and compared them to data acquired at another concentration, the highest p value among the three imaging conditions was 6.7×10^{-7}. Thus, the two concentrations were clearly distinguishable and the null hypothesis, which stated that both concentrations were equivalent, was rejected. We also compared the two closest subsets (2×10^5 copies/mL at 57 °C and 1×10^5 copies/mL at 63 °C) and calculated their p-value under each set of imaging conditions. The p-values were still below 0.05 for all three conditions.

Additionally, we used normal distributions as visual guides for data interpretation. We can use normal distributions instead of theoretical t-distributions because we determined standard

deviations from the data. As shown in Figure S5, there was no visible overlap between the data sets corresponding to the two concentrations.

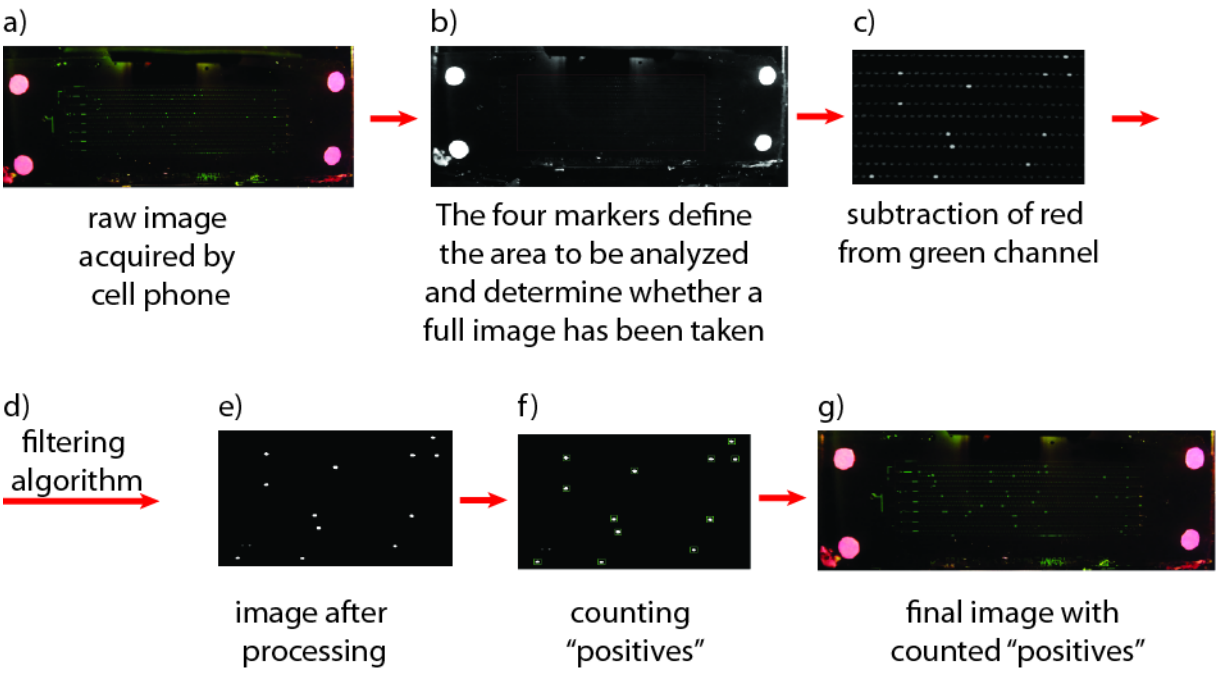


Supplementary Figure S5 Graphs showing normal distributions (calculated based on mean values and standard deviations) for two data sets at concentrations of 1 x 10^5 copies/mL and 2 x 10^5 copies/mL for dRT-LAMP. Three illumination conditions are shown: a) imaging with a microscope, b) imaging with a cell phone camera and shoe box, and c) imaging with a cell phone in dim lighting. Curves corresponding to the lower concentration (1 x 10^5 copies/mL) are shown

in blue, and curves corresponding to the higher concentration (2 x 10^5 copies/mL) are shown in violet. Mean numbers of positive counts with standard deviations are shown with arrows for clarity.

Cloud-based automatic analysis

The Symbian software on which the Nokia 808 cell phone is based can access Skydrive, a cloud-based storage service produced by Microsoft. This service can automatically upload images to the cloud for storage directly after imaging, without any user intervention. Almost instantly, those uploaded files are synced with all other computers running Skydrive that are logged in to the same account, and the images can be analyzed on multiple computers. Here, we used a secondary computer with a custom Labview program, including a built-in "file watcher" function whereby all files added to a specific directory that fell within a specific filtered category (i.e., *.jpg) were automatically analyzed. The analysis of images included a multi-step algorithm. First, the RGB color image was split into three monochrome 8-bit images for each individual color. The red-channel image was used to determine whether or not the entire chip had been imaged by searching for markers on the device (four red circles of tape, in this case). If all circles had been found, the image was then rotated such that the device was parallel to the top of the image box, removing any rotational bias. A background-corrected image was then generated by subtracting the red-channel monochrome image from the green-channel monochrome image, which contained the fluorescence information. The image was then subjected to a filtering process to increase the intensity of the positive wells. The filtering process included the following steps, in the following order: i) a 3 x 3 "local average" filter, ii) a 2 x 2 "median" filter, iii) an 11 x 11 "highlight details" filter, and iv) a 5 x 5 "median" filter. The filtered image was then thresholded using an entropy algorithm. After thresholding, a portion of the image (defined by the position of the markers) was analyzed and all individual spots were subjected to a size-filtering algorithm. This yielded the eventual total number of counts, which was then statistically transformed into a concentration before being emailed to the user or proper authority. The process detailing the image analysis is shown in Supplementary Figure S6, and a video detailing the process as seen by the user are also found in the Supplementary Information.



Supplementary Figure S6 A series of panels that show the image processing algorithm detailed at different steps of the process. a) A raw image acquired by a cell phone. b) The same image with blue and green channels subtracted, leaving only the red channel and thus showing the alignment markers that are used to check image fidelity and determine the area of analysis. c) An image for which background subtraction has been performed through subtraction of the red from the green channel. d) The image is processed by a multistep filtering algorithm before being thresholded with an entropy-based threshold algorithm. e) The image after processing and filtering. e-f) Thresholded images showing individual spots of fluorescence, or "positives," that are analyzed. The number of positive reactions is determined in order to quantify the concentration of the input.

Mathematical analysis of rate variations

To illustrate the errors of kinetic assays arising from variation in the rate constant of the autocatalytic process, an exponential amplification reaction can be described by the equation $x_f = x_i \times 2^{kt}$ where xf (unitless) is the final number of analyte molecules in the reaction volume, xi (unitless) is the initial number of analyte molecules, k [min-1] is a rate coefficient, and t [min] is the reaction time. Assuming as an example that the rate coefficient is equal to 1 [min-1], starting with 10 molecules of the analyte, and allowing the reaction to proceed for 24 minutes, there would be effectively 24 doublings resulting in 1.7 x 108 molecules of the analyte. For simplicity, consider 1.7 x 108 analyte molecules as the quantification threshold which, along with the reaction time, is used to back-calculate the starting number of the analyte molecules. In other words, using this value of the rate constant, by observing 1.7 x 108 molecules of the analyte at 24 minutes, one would conclude that the initial number of molecules was 10.

However, if the rate constant was reduced by 10%, 25%, and 50%, the actual numbers of molecules one would have to start with to obtain 1.7 x 108 molecules of the analyte at 24 minutes would be 52, 640, and 41000, corresponding to the errors of 420%, 6300%, and 410,000% respectively. Thus, when exponential amplification is used for detection, small temperature changes that affect the rate constants lead to large errors.

Video S1 A video demonstrating that a minimally trained user can use the approach described in this paper. After single molecules were amplified on SlipChip, a cell phone was used to image the chip in a shoebox. The image was then sent to a remote server, where the pattern of "positive" and "negative" wells on the chip was automatically analyzed, Poisson statistics was applied, and the number of molecules present in the sample was calculated. Quantitative results were automatically sent via email.

Supplementary References

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